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Modulation of Distinct Asthmatic Phenotypes in Mice by Dose-Dependent Inhalation of Microbial Products

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Running title: Modulation of immunity by inhaled microbial products

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Abstract

Background: Human asthmatics display considerable heterogeneity with regard to Th2-associated eosinophilic and Th17-associated neutrophilic inflammation, but the impact of the environment on these different forms of asthma is poorly understood.

Objective: We studied the nature and longevity of asthma-like responses triggered by inhalation of allergen together with environmentally relevant doses of inhaled LPS.

Methods: Ovalbumin was instilled into the airways of mice together with a wide range of LPS doses. Following a single OVA challenge, or multiple challenges, animals were assessed for pulmonary cytokine production, airway inflammation and airway hyperresponsiveness (AHR).

Results: Mice instilled with OVA together with very low doses ($\leq 10^{-3}$ μg) of LPS displayed modest amounts of Th2 cytokines, with associated airway eosinophilia and AHR after a single challenge, and these responses were sustained after multiple OVA challenges. When the higher but still environmentally relevant dose of 10^{-1} μg LPS was used, mice initially displayed similar Th2 responses, as well as Th17-associated neutrophilia. After multiple OVA challenges, however, the latter animals also accumulated large numbers of allergen-specific T regulatory (Treg) cells having high display of inducible co-stimulatory molecule (ICOS). As a result, asthma-like features in these mice were shorter-lived than in mice sensitized using lower doses of LPS.

Conclusions: The nature and longevity of Th2, Th17 and Treg immune responses to inhaled allergen are dependent on the quantity of LPS inhaled at the time of allergic sensitization. These findings might account in part for the heterogeneity of inflammatory infiltrates seen in lungs of asthmatics.

Introduction

Allergic asthma is a chronic disease of the airways characterized by airway inflammation, mucus production and reversible airway obstruction (Busse and Lemanske 2001). These features stem largely from the actions of T helper (Th) 2 cells, which produce the cytokines IL-4, IL-5 and IL-13 (Herrick and Bottomly 2003; Larche et al. 2003) and drive eosinophilic inflammation and mucus production. However, asthmatics display considerable heterogeneity with regard to inflammatory cells in the airway, and many patients have neutrophilic inflammation with little evidence of eosinophilia (McGrath et al. 2012). Unfortunately, these patients respond poorly to inhaled corticosteroids, the standard asthma therapy, and emerging data suggest that neutrophilic asthma might result from IL-17A (IL-17) production by steroid resistant, Th17 cells (Barczyk et al. 2003; Bullens et al. 2006; McKinley et al. 2008). Although IL-17 does not directly recruit neutrophils, it triggers airway epithelial cells to secrete neutrophil attracting chemokines, such as IL-8 (Laan et al. 1999). In addition to having allergen-specific Th2 and Th17 effector responses, asthmatics also appear to lack sufficiently strong regulatory mechanisms to keep effector responses in check (Akdis et al. 2004; Lloyd and Hawrylowicz 2009).

The increased prevalence of allergic asthma over the last several decades has been linked to exposure to several environmental factors, including bacteria and their products. Accordingly, the so-called ‘hygiene hypothesis’ postulates that increased hygiene, smaller families, and consequent decreased exposure to pathogens is at least partially responsible for the increase in allergic diseases (Strachan 1989). It was initially proposed that infections protect against Th2-related diseases by skewing immunity towards Th1 responses, but more recent evidence suggests that increased regulatory responses are responsible for this protective effect (Yazdanbakhsh et al.

2002). It is currently unclear whether the quantities of bacterial products in natural environments impact the balance of effector and regulatory responses to inhaled allergens.

Lipopolysaccharide (LPS), a major component of the outer cell wall of Gram-negative bacteria, is ubiquitous in the environment, and some studies have indicated that exposure to relatively high levels of LPS during childhood protect against developing asthma later in life (Braun-Fahrlander et al. 2002; El-Sharif et al. 2006; Gehring et al. 2004). Conversely, other studies have demonstrated a positive association between asthma and household levels of bacteria and LPS (Ross et al. 2000; Thorne et al. 2005). Eisenbarth and colleagues previously reported that in mice, a large amount of inhaled LPS (10 μg) promotes Th1 responses, whereas a smaller amount (100 ng) promotes Th2 responses. Subsequent studies showed that LPS can also promote Th17 responses in the lung (Wilson et al. 2009) and intestine (McAleer et al. 2010). Paradoxically, LPS can also induce T regulatory (Treg) cell expansion directly (Caramalho et al. 2003), or indirectly by activating IL-10-producing dendritic cells (Lau et al. 2008). In most such experiments, relatively large amounts of LPS were used and the impact of more environmentally relevant amounts of LPS on immune responses is unclear. Finally, it is not known whether inhaled LPS impacts immune responses to intermittent allergen exposures, as well as responses to chronic exposures such as those occurring in the home or work environments. Accordingly, the current study was initiated to test the hypothesis that different amounts of inhaled LPS can not only trigger different forms of asthma, but also modulate regulatory responses that control the actions of allergen-specific, effector T cells. We used established mouse models of asthma based on a highly purified form of ovalbumin (OVA), which lacks detectable LPS and is non-allergenic in the absence of exogenously added adjuvants (Eisenbarth et al. 2002; Wilson et al. 2009). One model mimicked intermittent exposures and the other mimicked more chronic

exposures. Herein, we show that extremely low doses of inhaled LPS are sufficient to prime immune responses to OVA, and that these responses lead to sustained airway eosinophilia and AHR. Higher, but still environmentally relevant doses of LPS induce both Th2 and Th17 responses in regional lymph nodes (LNs). In addition, regulatory responses are induced and as a result, asthma-like responses in these animals are consequently short-lived. Thus, levels of microbial products determine the balance of Th2, Th17 and regulatory responses to either promote or suppress the asthmatic phenotype. We also found that the amounts of LPS found naturally in house dust are sufficient to confer both Th17 and Treg responses in a dose dependent manner. These findings suggest that differing levels of environmental LPS in previous studies might explain discordant conclusions regarding the effect of environmental endotoxin on allergic responses.

Materials and Methods

Mice

C57BL/6J, BALB/cJ, *Foxp3*^{gfp}, OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J) and CD45.1 (B6.SJL-*Ptprca Pepcb*/BoyJ) mice were purchased from Jackson Laboratories, Bar Harbor, ME. The latter two strains were crossed to one another. *Tlr4*$^{-/-}$ mice were obtained from Shizuo Akira (Osaka University, Japan). Mice were housed at a maximum of 3 animals per 12.9”L X 7.5”W X 5.6”H polysulfone, static micro-isolator cage (Lab Products) in specific pathogen-free conditions at the NIEHS and used between 6 and 12 weeks of age. Paper bedding (Diamond Soft: Harlan Teklad) was used to minimize inhaled dust, and the cages were changed twice weekly. All animals were treated humanely and with regard for alleviation of suffering. All animal

experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the NIEHS.

Adoptive transfer of OT-II T cells and analysis of cytokines in regional LNs

$CD4^+$ T cells were prepared as previously described (Whitehead et al. 2011) from (CD45.1) OT-II mice, which carry a transgene encoding a T cell receptor specific for OVA, and were transferred by retroorbital injection into (CD45.2) C57BL/6J mice. Four hours later, recipient mice were sensitized to OVA using LPS as an adjuvant. Four days later, mediastinal LNs were excised, minced, pressed through a 70 μm strainer and cultured at a concentration of 5×10^5 in 100 μl RPMI with 10% FBS and 10 μg/mL OVA for 2 days. Culture supernatants analyzed for cytokines as described above.

LPS- and HDE-mediated allergic airway disease

Mice were anesthetized with inhaled isoflurane, and sensitized in the mornings of days 0 and 7 by oropharyngeal instillations (Wilson et al. 2009) of LPS-depleted OVA (BioVendor Inc. Candler, NC, cat# 321000) together with various amounts of *E. coli* LPS (Sigma, St. Louis, MO, cat# l2630), HDEs or LPS-supplemented HDEs. Asthma-like responses were elicited in the morning by exposing sensitized animals to nebulized (Ultra-Neb99, DeVilbiss healthcare, Somerset, PA) 1% OVA (Sigma, St. Louis, MO, cat# A5503) in saline, either on a single occasion (day 14) for 1 hour, or daily for 30 minutes on 6 consecutive days (day14 – 19). Separate groups of mice were used to measure peak cellular inflammation and AHR, which corresponded to 48 hours after a single challenge, and 24 hours after multiple challenges. For AHR, mice were anesthetized by i.p. injections of urethane (1.5 g/kg) and paralyzed with i.p. injections of pancuronium bromide (0.8 mg/kg), intubated and analyzed using the FlexiVent

mechanical ventilator system (Scireq, Montreal, PQ, Canada) as previously described (Wilson et al. 2009). The single compartment model of the lung was used to assess total respiratory system resistance (R) after delivery of aerosolized methacholine (0 to 50 mg/ml for C57BL/6 mice; 0 to 25 mg/ml for BALB/cJ mice) through an ultrasonic nebulizer. Peak resistance values are reported. Cell differential analysis of cells in the BAL was performed as described previously (Hollingsworth et al. 2004). For cytokine analysis, whole lung bronchoalveolar lavage (BAL) was performed 4 hours post-challenge, and IL-4, IL-5, IL-17A and IFN-γ were measured using a multiplexed fluorescent bead-based immunoassay (Bio-Rad laboratories, Hercules, CA). To assess antibody responses, venous blood was collected and serum was separated via plasma separator tubes and centrifugation. Murine IgE ELISA kit (BD Biosciences, San Diego, CA) was used to quantify IgE in the serum samples that were diluted 1:20. BAL was performed to assess cellular inflammation.

House dust extracts (HDEs)

HDEs were prepared as described previously (Sever et al. 2007). Endotoxin levels were assayed by a limulus amebocyte lysate (LAL) assay (Lonza, Karlsruhe, Germany) and allergens were measured using a multiplex array for indoor allergens (MARIA) (Indoor Biotechnologies, Charlottesville, VA), according to procedures provided by the manufacturer.

Flow cytometry

Fluorochrome labeled antibodies against mouse CD4 (RM4-5), CD45.1 (A20), Foxp3 (FJK-16s), and ICOS (C398.4A) were purchased from eBioscience (San Diego, CA). For analysis of OVA-specific Tregs in the lung, mice receiving adoptive transfers of OT-II cells were sensitized with OVA together with various amounts of LPS and challenged on days 7-12 with 1% OVA aerosol

for 30 minutes. Lungs were excised on day 13, minced and enzymatically digested as previously described (Nakano et al. 2011), and CD4+ FOXP3+ containing Tregs stained with antibodies against ICOS (Wilson et al. 2009). Cells were run on an LSR II flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using Flowjo software (Treestar, Ashland, OR).

Statistics

Statistical differences between multiple groups were first calculated using a one-way ANOVA. To compare mice treated OVA together with different doses of LPS to control mice treated with OVA only, a post-hoc Dunnett's multiple comparison test was used. To compare mice treated with OVA plus 10^{-1} μg LPS to lower doses, a post-hoc Student's *t* test was used. When comparing only two groups to one another, a Student's *t* test was used. Analyses were done using GraphPad Prism v5.02 (GraphPad Software, Inc., San Diego, CA). *P*-values of less than 0.05 were considered statistically significant.

Results

Effect of LPS dose on T cell priming in draining LNs

We first investigated the impact of inhaled LPS dose on the initiation of antigen-specific T cell priming, which occurs in regional LNs. OVA-specific T cells from (CD45.1) OT-II mice were transferred into recipient C57BL/6 mice, and these animals were allowed to inhale highly purified OVA together with various amounts of LPS. These doses ranged in 100-fold increments, and included the previously reported doses of 10 μg and 10^{-1} μg (Eisenbarth et al. 2002; Wilson et al. 2009), as well as much smaller doses, down to 10^{-7} μg. On the day after OVA/LPS inhalation, cells from excised regional LNs were incubated with OVA, and the culture

supernatants analyzed for cytokines diagnostic of different T helper cell lineages. The concentration of the Th2 cytokines, IL-4 and IL-5, in supernatants of these cultures increased in concert with the dose of LPS used during the sensitization, except that almost no IL-4 or IL-5 was detected when the highest dose of 10 μg LPS was used as an adjuvant (Figure 1). IL-17 production in regional LNs also increased with the dose of LPS used during the sensitization, including 10 μg LPS. The concentrations of IFN-γ also increased with the amount of inhaled LPS, including the highest dose of 10 μg LPS, in agreement with previous reports that high doses of LPS prime Th1 responses (Eisenbarth et al. 2002).

Impact of LPS dose during sensitization on cytokines in the lung and on serum IgE

We next determined whether the production of cytokines in the lung following OVA sensitization and challenge would follow the same trend as those produced in regional LNs after sensitization. Mice were sensitized to OVA using various doses of LPS and subsequently exposed to aerosolized OVA on a single occasion to mimic intermittent allergen exposures, or on 6 consecutive days to mimic more chronic exposures (Figure 2A). After a single challenge, mice previously given OVA without LPS, or with the very high dose of 10 μg LPS, had very low amounts of IL-5 in the airway (Figure 2B), consistent with the low amounts of this cytokine seen in regional LNs of similarly sensitized mice (Figure 1). Mice sensitized with either 10^{-3} μg or 10^{-1} μg LPS had much higher amounts of IL-5 in the airway after OVA challenge, again reflecting the increased amounts of this cytokine seen in regional LNs. After 6 challenges, the amounts of IL-5 in BAL were lower than after a single challenge, and the trend generally correlated well with the amounts we had observed in regional LNs. Notably, an exception to this trend was mice that had been sensitized to OVA using 10^{-1} μg LPS and challenged on six occasions. Despite having very high levels of IL-5 in regional LNs and also in the airway after a single challenge,

these animals had lower amounts of IL-5 in the airway than mice sensitized using lower amounts of LPS. IL-4, a Th2 cytokine that promotes immunoglobulin class switching to IgE, was lower after multiple challenges than after a single challenge, but was not lower in mice sensitized with 10^{-1} μg LPS than in mice sensitized using 10^{-3} μg LPS, indicating that IL-4 and IL-5 are differentially regulated.

We also analyzed production of IL-17 in the airways of challenged mice because it can promote neutrophil recruitment and AHR in this murine model of asthma (Wilson et al. 2009). As expected, almost no IL-17 was seen in mice that inhaled OVA without LPS. However, the concentrations of this cytokine in the airway increased in proportion to the amount of LPS used during sensitization. After six challenges, IL-17 levels were reduced compared to after a single challenge, but the trend of increased IL-17 with increased LPS during sensitization was maintained. Thus, unlike IL-5, the amount of IL-17 in the lung after challenge was closely associated with levels of that cytokine in draining LNs across the entire range of LPS doses tested. IFN-γ, the signature cytokine of Th1 cells, was not significantly different among the various groups, suggesting that at these doses, increased Th1 responses do not account for the observed reduction in Th2 responses.

Atopic diseases are associated with elevated levels of IgE antibodies, which bind to Fc receptors on the surface of several cell types, including mast cells and basophils. We therefore studied total serum IgE in mice that had been sensitized to OVA using different amounts of LPS. We found that titers of this isotype increased in concert with amount of LPS used during sensitization (Figure 2C). The single exception to this pattern was mice that had been sensitized using 10 mg of LPS and challenged on six occasions. These animals trended towards lower levels of IgE than similarly challenged animals that had been sensitized using 10^{-1} μg LPS.

Dose of LPS during allergic sensitization determines the nature of allergen-induced pulmonary inflammation

We next studied the impact of LPS dose during sensitization on the types of leukocytes that accumulate in the airway following OVA challenge on a single occasion, or on 6 consecutive days. As expected, C57BL/6 mice that inhaled OVA only, or LPS only, had very few inflammatory cells in the airway after a single OVA challenge (Figure 3 and data not shown). This suggests that levels of LPS in the home cages are not sufficient to act as a strong adjuvant for OVA sensitization. However, mice receiving OVA together with as little as 10^{-7} μg LPS developed low, but measurable airway eosinophilia after a single challenge, and this response was strongly increased after six daily challenges (Figure 3). Surprisingly, the strongest eosinophilic response was seen in mice that had been sensitized using only 10^{-5} μg LPS. Mice sensitized to OVA using 10^{-1} μg LPS had markedly fewer eosinophils than mice sensitized using lower amounts of LPS, despite having the highest amounts of IL-4 and IL-5 in lung-draining lymph nodes (Figure 1). Similar results were obtained for BALB/c mice (see Supplemental Material, Figure S1), ruling out the possibility that our findings were unique to C57BL/6 mice. The low numbers of eosinophils in animals sensitized to OVA using 10^{-1} μg LPS were consistent with the correspondingly low concentrations of IL-5 in airways of these animals after six OVA challenges (Figure 2B). Together, these observations suggest that although very low doses of LPS prime relatively weak Th2 responses in the draining lymph node, they lead to persistent airway eosinophilia, whereas the moderate dose of 10^{-1} μg LPS initially triggers stronger Th2 responses, but shorter-lived pulmonary eosinophilia.

In contrast to our findings for eosinophil accumulation, the number of neutrophils in the airway following OVA challenge was proportional to the amount of LPS given during OVA

sensitization, and were not markedly increased by more OVA challenges. Generally, the number of neutrophils correlated well with airway levels of IL-17. Interestingly, although LPS during sensitization was required for lymphocyte infiltration after OVA challenge, increased amounts of LPS did not lead to increased lymphocytes. By contrast higher amounts of LPS during sensitization generally led to more macrophages in the airway.

Effect of LPS dose during sensitization on subsequent OVA challenge-induced AHR

AHR is a cardinal feature of asthma. Accordingly, we used invasive measurements of airway resistance to study the effect of LPS dose during allergic sensitization on the development and progression of AHR. As expected, mice that received OVA alone during the sensitization phase did not develop AHR after a single OVA challenge or after six challenges. By contrast, mice sensitized using doses of LPS ranging from 10^{-7} to 10^{-1} μg displayed increased airway resistance in response to methacholine after a single OVA challenge (Figure 4, and Supplemental Material, Figure S2A). This AHR was sustained in mice that had been sensitized using very low amounts of LPS, but not in mice sensitized using 10^{-1} μg LPS. Similar results were obtained when BALB/c mice were examined (Supplemental Material, Figure S2B). Thus, like eosinophilic inflammation, AHR was also sustained after multiple OVA challenges in mice sensitized using very low doses of LPS, but not in mice sensitized using the higher, but still moderate dose of 10^{-1} μg LPS.

Environmental amounts of LPS are sufficient to prime both Th17 and regulatory responses to inhaled allergens

We next investigated whether the amounts of LPS found in natural environments are capable of priming Th2 and Th17 responses that lead to airway eosinophilia, neutrophilia and AHR. We

reasoned that common house dust would provide a good representation of indoor environments. We therefore prepared extracts from dust samples and tested their abilities to promote allergic responses to co-instilled OVA. Two HDEs were evaluated; both contained dust mite allergens, but differed in their endotoxin activity (see Supplemental Material, Figure S3A). One HDE had a relatively low endotoxin activity of 2,000 EU/ml, approximately equal to 10^{-2} μg LPS / 20 μl HDE ('Endo lo'). The other HDE had a higher endotoxin activity approximately equal to 10^{-1} μg LPS / 20 μl HDE ('Endo mod'). In a dose response experiment, we found that neutrophilic and eosinophilic responses after OVA challenge generally increased with increasing doses of HDE used during sensitization, and that at low doses, the 'Endo mod' HDE was more effective than the 'Endo lo' HDE (Figure 5A). However, mice receiving 20 μl of the 'Endo mod' HDE during sensitization had fewer airway eosinophils after the 6-day challenge mice than mice sensitized using lower amounts of this extract. This observation was reminiscent of the reduced airway eosinophilia in mice sensitized using 10^{-1} μg LPS and challenged on six occasions, compared with mice sensitized using lower amounts of this microbial product (Figure 3).

HDEs are complex mixtures and typically contain multiple adjuvants and allergens (see Supplemental Material, Figure S3B). To confirm that moderate amounts of LPS in house dust can reduce Th2 responses and eosinophilic inflammation, we carried out two additional experiments. First, we studied the 'Endo mod' HDE in *Tlr4*-deficient mice, which are unable to respond to LPS. These *Tlr4*-deficient mice displayed very little neutrophilic inflammation after OVA challenge, but had increased eosinophils (Figure 5B). This result suggests that LPS residing in HDE promotes adaptive immune responses that drive neutrophil accumulation in the lung and suppress eosinophil accumulation. To confirm this, we added 10^{-1} μg of exogenous LPS to the 'Endo lo' HDE and tested its adjuvant activity. This addition of LPS led to significantly

reduced eosinophils in the airway, particularly in mice that had been challenged on six occasions (Figure 5C).

Effect of LPS dose during sensitization on OVA-specific and nonspecific Tregs in the chronic asthma model

We next investigated mechanisms that might be responsible for the observed reduction in eosinophils seen after multiple exposures in mice sensitized using moderate amounts of LPS. Because pulmonary levels of IFN-γ were not significantly increased in mice sensitized using 10^{-1} μg of LPS compared with mice sensitized using lower amounts, it seemed unlikely that Th1 responses were entirely responsible for suppressing eosinophilic inflammation. Moreover, analysis of draining lymph nodes had revealed that Th2 cytokines were highest when 10^{-1} μg of LPS was used during sensitization. We considered the possibility that inhaled LPS induces regulatory responses that gain in strength after multiple challenges and suppress eosinophilic inflammation. In some murine models of asthma, IL-10 can suppress allergic inflammation in the lung (Joetham et al. 2007; Kearley et al. 2005; Oh et al. 2002), but amounts of this cytokine were similar in lungs of mice sensitized using high or low doses of LPS (data not shown). We therefore studied Forkhead box protein 3 $(Foxp3)^+$ $CD4^+$ T cells regulatory T cells (Tregs), which can effectively suppress established allergic responses (Lloyd and Hawrylowicz 2009), including those induced by OVA/LPS (Whitehead et al. 2011). Two main types of Tregs have been identified; natural (n)Tregs that develop in the thymus and recognize self-antigens, and induced (i)Tregs that develop in the periphery and recognize exogenous antigens. These two cell types have overlapping, but distinct activities and can be distinguished by their transcriptional profile (Bilate and Lafaille 2012). We first evaluated total Tregs in the lung using reporter mice in which expression of a green fluorescent protein-encoding gene (*gfp*) is controlled by

transcriptional control elements of the *Foxp3* gene. We found that total GFP^+ $CD4^+$ Tregs were present at similar levels in the lung whether 10^{-3} μg LPS or 10^{-1} μg LPS was used in the sensitization phase (Figure 6A). These experiments ruled out total Treg cell number as being the sole factor responsible for suppression, but left open the possibility that allergen-specific, iTregs might be particularly important in this regard. To investigate this possibility, we adoptively transferred OVA-specific (CD45.1) OT-II cells into (CD45.2) recipient mice prior to their sensitization and challenged the animals on 6 consecutive days. Recipient $CD4^+$ T cells comprised the vast majority of total $CD4^+$ T cells and their numbers were increased in OVA/LPS sensitized mice compared with mice treated with PBS or 'OVA only' (Figure 6B, and data not shown). The number and percentages of recipient $Foxp3^+$ Tregs were similar in mice sensitized using 10^{-3} μg LPS or 10^{-1} μg LPS (Figure 6C), but OVA-specific, CD45.1 $CD4^+$ donor T cells, including $Foxp3^+CD4^+$ Tregs, were more abundant in mice sensitized using 10^{-1} μg LPS than in mice sensitized using 10^{-3} μg LPS (Figure 6C, data not shown). This suggested that inhalation of 10^{-1} μg LPS might, after multiple OVA challenges, lead to suppression of allergic responses by increasing the number of OVA-specific $Foxp3^+CD4^+$ iTregs in the lung.

Tregs are heterogeneous cells that might have functional differences. For example, $Foxp3^+$ $CD4^+$ nTregs in humans are comprised of populations with distinct cell surface display levels of inducible T cell co-stimulator (ICOS) (Ito et al. 2008). Previous studies have shown that suppression of airway inflammation and AHR is associated with high levels of ICOS expression on $CD4^+$ Tregs (Whitehead et al. 2011), and that adoptive transfer of $ICOS^+$ $CD4^+$ T cells, but not $ICOS^-$ cells, suppresses established AHR in mice (Shalaby et al. 2012). We therefore compared numbers of $ICOS^+Foxp3^+$ Tregs, as well as the amount of ICOS on the cell surface of Tregs, in mice sensitized using 10^{-3} μg or 10^{-1} μg LPS. The total numbers of recipient

$ICOS^+Foxp3^+$ Tregs were similar in mice from these two groups (Figure 6D). However, the number of OVA-specific, donor $ICOS^+$ $Foxp3^+$ Tregs, as well as the intensity of ICOS staining on these cells, were higher in mice sensitized using the suppressive dose of 10^{-1} μg LPS than in those sensitized with the non-suppressive dose of 10^{-3} μg. This suggests that moderate doses of inhaled LPS might lead to suppression of established allergic responses in the chronic challenge model by increasing the number of $ICOS^+Foxp3^+$ Tregs and the levels of ICOS on these cells.

Discussion

It is widely accepted that environmental endotoxin can impact the prevalence and severity of allergic asthma. However, whereas some studies have shown a positive association between endotoxin and asthma, others have suggested that LPS protects against developing this disease. In mice, very high doses of inhaled LPS have been previously reported to promote Th1 responses, with lower doses promoting Th2 responses. However, the impact of LPS levels on Th17 and T regulatory cells (Tregs) has not been extensively studied. This issue has become increasingly important because these two cell types are now thought to have critical roles in some forms of asthma, including non-eosinophilic asthma. Our current findings shed light on this issue by showing that the nature and longevity of immune responses to inhaled OVA are remarkably sensitive to the amounts of LPS inhaled at the time of sensitization. The lowest dose of LPS that we tested (10^{-7} μg) is one million times lower than the 10^{-1} μg used previously in this model to elicit allergic sensitization to inhaled OVA (Eisenbarth et al. 2002; Wilson et al. 2009). Although effective even at extremely low concentrations, some LPS was necessary for sensitization because mice receiving OVA with no LPS failed to become sensitized, as judged by their lack of allergic responses to subsequent OVA challenge. It should be noted that our mice

are housed in conditions designed to minimize pulmonary inflammation from dust or endotoxin, and it is possible that mice house in dirtier conditions might have responded differently.

Reported amounts of endotoxin in the environment vary according to the location, season, and methods used for endotoxin detection, and depend on whether dust, defined particulate matter or ambient air is measured. Ambient air contains endotoxin that typically ranges from 0.1 to 4 EU/m^3 (Tager et al. 2010), although endotoxin levels can reach up to 50,000 EU/m^3 in some industrial settings, such as swine containment facilities (Dungan 2011). In the experiments described here, experimental mice were instilled with a liquid containing from 0.001 EU (10^{-7} μg LPS) to 100,000 EU (10 μg LPS), spanning the range of doses encountered in the environment. The ability of extremely low doses of LPS to prime allergic responses suggests that the amounts of this bacterial product found in the environment are sufficient to promote allergic responses to environmental allergens. However, continuous exposure to low amounts of LPS in ambient air is likely quite different than intermittent exposures to higher doses, even if the cumulative doses are similar.

It was previously reported that 10^{-1} μg of inhaled LPS promotes Th2 responses, whereas the much higher dose of 100 μg LPS promotes Th1 responses (Eisenbarth et al. 2002). These observations led to the hypothesis that a threshold level of LPS switches Th2 responses to Th1 responses (Vercelli 2003). Our current data are in partial agreement with this hypothesis because we found that in draining lymph nodes, the highest level of LPS used here (10 μg) promoted Th1 responses – and Th17 responses – at the expense of Th2 responses. However, our findings also suggest that the relationship between environmentally relevant amounts of LPS and immune responses is much more complex than this. At low doses of LPS (10^{-1} μg or less), increasing amounts of LPS during sensitization led to increases in Th2 cytokines in draining lymph nodes,

as well as increases in Th1 and Th17 cytokines. Thus, in contrast to a widely held belief, environmentally relevant concentrations of LPS do not inhibit the initiation of Th2 responses in draining lymph nodes. However, mice sensitized using the moderate dose of 10^{-1} μg LPS had markedly less IL-5 in the airway, and had less eosinophilia and AHR after 6 challenges than mice sensitized using lower amounts of LPS. It seems unlikely that this reduction in asthma-like features is due entirely to a switch from Th2 to Th1 responses because we did not observe increased airway IFN-γ in mice sensitized using 10^{-1} μg LPS and because for doses up to 10^{-1} μg increasing concentrations of LPS led to stronger Th2 responses in draining lymph nodes. We therefore propose that 10^{-1} μg of LPS induces regulatory responses that suppress Th2 and Th17 cell actions, particularly after multiple allergen challenges. We have previously shown that the numbers of Tregs in the lung increases with the number of OVA challenges and that depletion of $Foxp3^{+}$ Tregs increases allergic inflammation and AHR (Whitehead et al. 2011). In the present study, we found that OVA-specific iTregs were more abundant in mice sensitized using the suppressive dose of 10^{-1} μg LPS than in mice receiving the non-suppressive dose of 10^{-3} μg LPS. This increase in Treg number was partly due to a corresponding increase in total OVA-specific T cells, but a threshold number of Tregs in the lung might be required for suppression after multiple challenges. It is also possible that OVA specific iTregs have a greater impact on pulmonary inflammation than nTregs, although fewer in number.

TLR4 is present on the surface of at least some Tregs, and LPS can promote expansion of these cells (Caramalho et al. 2003). However, in the experiments described here, pre-existing OVA-specific iTregs would be unlikely because the animals had not previously been exposed to OVA. Thus, the LPS instilled together with OVA during the sensitization phase likely functioned to promote the development of these cells, which probably proliferated during the challenge phase.

Cell surface display levels of ICOS on $Foxp3^+$ Tregs were also highest in mice sensitized using the suppressive dose of 10^{-1} μg LPS as an adjuvant. We did not directly compare the function of $ICOS^{hi}$ and $ICOS^{lo}$ Tregs in the present study, but it has been previously shown that ICOS is required for suppression of allergic responses (Whitehead et al. 2011) and that adoptive transfer of $ICOS^+$ $CD4^+$ T cells suppresses airway eosinophilia and AHR, whereas transfer of $ICOS^-$ $CD4^+$ T cells does not (Shalaby et al. 2012).

The LPS used in the current study likely contains other microbial products that might have contributed to the observed immune and inflammatory responses. Similarly, the composition of HDEs is also highly complex and undoubtedly contains microbial products and allergens, in addition to LPS. However, an important advantage of using HDEs is that they are derived from typical indoor environments and therefore more accurately reflect natural exposures than does instillation of arbitrary amounts of any single microbial product. It is therefore important that addition of LPS to the 'endo lo' HDE was sufficient to dramatically reduce the ability of that extract to promote sustained airway eosinophilia after six OVA challenges.

In summary, our findings demonstrate that immune responses to inhaled allergens are highly dependent on the doses of inhaled LPS, including the amounts found naturally in the environment. We propose that exposure to relatively low doses of LPS promotes classical, Th2-driven allergic responses to inhaled allergens, whereas moderate doses of this microbial product induce stronger Th17 responses and associated neutrophilia. In addition, inhalation of moderate doses of LPS during sensitization induces regulatory responses that, after multiple allergen exposures, limit the severity and longevity of asthma-like features.

References

Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific t regulatory 1 and t helper 2 cells. J Exp Med 199:1567-1575.

Barczyk A, Pierzchala W, Sozanska E. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med 97:726-733.

Bilate AM, Lafaille JJ. 2012. Induced cd4+foxp3+ regulatory t cells in immune tolerance. Annu Rev Immunol 30:733-758.

Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. 2002. Environmental exposure to endotoxin and its relation to asthma in school-age children. The New England Journal of Medicine 347:869-877.

Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, et al. 2006. Il-17 mrna in sputum of asthmatic patients: Linking t cell driven inflammation and granulocytic influx? Respir Res 7:135.

Busse WW, Lemanske RF, Jr. 2001. Asthma. N Engl J Med 344:350-362.

Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. 2003. Regulatory t cells selectively express toll-like receptors and are activated by lipopolysaccharide. The Journal of Experimental Medicine 197:403-411.

Dungan RS. 2011. Airborne endotoxin from indoor and outdoor environments: Effect of sample dilution on the kinetic limulus amebocyte lysate (lal) assay. J Occup Environ Hyg 8:147-153.

Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent t helper cell type 2 responses to inhaled antigen. J Exp Med 196:1645-1651.

El-Sharif N, Douwes J, Hoet P, Nemery B. 2006. Childhood asthma and indoor aeroallergens and endotoxin in palestine: A case-control study. The Journal of Asthma: Official Journal of the Association for the Care of Asthma 43:241-247.

Gehring U, Bischof W, Schlenvoigt G, Richter K, Fahlbusch B, Wichmann HE, et al. 2004. Exposure to house dust endotoxin and allergic sensitization in adults. Allergy 59:946-952.

Herrick CA, Bottomly K. 2003. To respond or not to respond: T cells in allergic asthma. Nat Rev Immunol 3:405-412.

Hollingsworth JW, 2nd, Cook DN, Brass DM, Walker JK, Morgan DL, Foster WM, et al. 2004. The role of toll-like receptor 4 in environmental airway injury in mice. American Journal of Respiratory and Critical Care Medicine 170:126-132.

Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, et al. 2008. Two functional subsets of foxp3+ regulatory t cells in human thymus and periphery. Immunity 28:870-880.

Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T, et al. 2007. Naturally occurring lung cd4(+)cd25(+) t cell regulation of airway allergic responses depends on il-10 induction of tgf-beta. J Immunol 178:1433-1442.

Kearley J, Barker JE, Robinson DS, Lloyd CM. 2005. Resolution of airway inflammation and hyperreactivity after in vivo transfer of cd4+cd25+ regulatory t cells is interleukin 10 dependent. J Exp Med 202:1539-1547.

Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, et al. 1999. Neutrophil recruitment by human il-17 via c-x-c chemokine release in the airways. Journal of Immunology 162:2347-2352.

Larche M, Robinson DS, Kay AB. 2003. The role of t lymphocytes in the pathogenesis of asthma. J Allergy Clin Immunol 111:450-463; quiz 464.

Lau AW, Biester S, Cornall RJ, Forrester JV. 2008. Lipopolysaccharide-activated il-10-secreting dendritic cells suppress experimental autoimmune uveoretinitis by mhcii-dependent activation of cd62l-expressing regulatory t cells. Journal of Immunology 180:3889-3899.

Lloyd CM, Hawrylowicz CM. 2009. Regulatory t cells in asthma. Immunity 31:438-449.

McAleer JP, Liu B, Li Z, Ngoi SM, Dai J, Oft M, et al. 2010. Potent intestinal th17 priming through peripheral lipopolysaccharide-based immunization. J Leukoc Biol 88:21-31.

McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, et al. 2012. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. American Journal of Respiratory and Critical Care Medicine 185:612-619.

McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. 2008. Th17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J Immunol 181:4089-4097.

Nakano H, Free ME, Whitehead GS, Maruoka S, Wilson RH, Nakano K, et al. 2011. Pulmonary cd103(+) dendritic cells prime th2 responses to inhaled allergens. Mucosal Mmmunology.

Oh JW, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. 2002. Cd4 t-helper cells engineered to produce il-10 prevent allergen-induced airway hyperreactivity and inflammation. J Allergy Clin Immunol 110:460-468.

Ross MA, Curtis L, Scheff PA, Hryhorczuk DO, Ramakrishnan V, Wadden RA, et al. 2000. Association of asthma symptoms and severity with indoor bioaerosols. Allergy 55:705-711.

Sever ML, Arbes SJ, Jr., Gore JC, Santangelo RG, Vaughn B, Mitchell H, et al. 2007. Cockroach allergen reduction by cockroach control alone in low-income urban homes: A randomized control trial. J Allergy Clin Immunol 120:849-855.

Shalaby KH, Jo T, Nakada E, Allard-Coutu A, Tsuchiya K, Hirota N, et al. 2012. Icos-expressing cd4 t cells induced via tlr4 in the nasal mucosa are capable of inhibiting experimental allergic asthma. Journal of Immunology 189:2793-2804.

Strachan DP. 1989. Hay fever, hygiene, and household size. BMJ 299:1259-1260.

Tager IB, Lurmann FW, Haight T, Alcorn S, Penfold B, Hammond SK. 2010. Temporal and spatial patterns of ambient endotoxin concentrations in fresno, california. Environ Health Perspect 118:1490-1496.

Thorne PS, Kulhankova K, Yin M, Cohn R, Arbes SJ, Jr., Zeldin DC. 2005. Endotoxin exposure is a risk factor for asthma: The national survey of endotoxin in united states housing. Am J Respir Crit Care Med 172:1371-1377.

Vercelli D. 2003. Learning from discrepancies: Cd14 polymorphisms, atopy and the endotoxin switch. Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology 33:153-155.

Whitehead GS, Wilson RH, Nakano K, Burch LH, Nakano H, Cook DN. 2011. Il-35 production by inducible costimulator (icos)-positive regulatory t cells reverses established il-17-dependent allergic airways disease. The Journal of Allergy and Clinical Immunology.

Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. 2009. Allergic sensitization through the airway primes th17-dependent neutrophilia and airway hyperresponsiveness. American Journal of Respiratory and Critical Care Medicine 180:720-730.

Yazdanbakhsh M, Kremsner PG, van Ree R. 2002. Allergy, parasites, and the hygiene hypothesis. Science 296:490-494.

FIGURE LEGENDS

Figure 1. Impact of inhaled LPS on T cell priming in regional LNs. Mice received adoptive transfer of OVA-specific T cells, were sensitized to OVA using the indicated amounts of LPS as adjuvant, and the indicated cytokines were measured in cultures of mediastinal LNs collected 24 h post-sensitization. Data shown represent the mean cytokine concentration ± SEM, and are from one of 2 experiments yielding similar results. n = 6 mice/group. *a*, $P < 0.05$, OVA-LPS sensitized mice vs. 'OVA only' controls.

Figure 2. Effect of inhaled LPS dose during allergen sensitization on cytokine production in the lung following allergen challenge of C57BL/6 mice. (A) Timeline of sensitization, challenge(s) and harvest. (B) Mean peak cytokine concentrations ± SEM in BAL of naïve mice (N) or mice sensitized to OVA using the indicated amounts of LPS and challenged to aerosolized OVA on a single occasion, or on six consecutive days. Limits of detection were IL-4, 2.1 pg/ml; IL-5, 0.3 pg/ml; IL-17, 0.8 pg/ml and IFN-γ 1.2 pg/ml. (C) Total IgE in sera from mice sensitized and challenged as indicated. Limit of detection; 0.016 μg/ml. Data are from one of 2 independent experiments giving similar results. n = 6 mice/group. *a*, $P < 0.05$, OVA-LPS sensitized mice vs. 'OVA only' controls; *b*, $P < 0.05$, 10^{-1} μg LPS vs. lower dose LPS groups.

Figure 3. LPS dose during sensitization differentially impacts leukocyte subset recruitment to the lung following allergen challenge. Mice sensitized to OVA using the indicated amounts of LPS were challenged to aerosolized OVA on a single occasion, or on six consecutive days. Data represent mean cell numbers ± SEM in the BAL of challenged, or naïve mice (N), and are from a single experiment representative of 3 independent experiments giving qualitatively similar

results. $n = 6$ mice/group. *a*, $P < 0.05$, OVA-LPS sensitized mice vs. 'OVA only' controls; *b*, $P < 0.05$, 10^{-1} µg LPS vs. lower dose LPS groups.

Figure 4. Effect of inhaled LPS dose during sensitization on airway responses to subsequent allergen challenge. Airway resistance (R) was measured in anesthetized mice that were sensitized to OVA using the indicated amounts of LPS, and challenged with aerosolized OVA on a single occasion, or on six consecutive days. Data shown represent the mean of peak R values ± SEM following inhalation of 50 mg/ml methacholine (MCH). Data are from one of 2 experiments giving similar results. $n = 6$ mice/group. *a*, $P < 0.05$, OVA-LPS sensitized mice vs. 'OVA only' controls; *b*, $P < 0.05$, 10^{-1} µg LPS vs. lower dose LPS groups.

Figure 5. Contribution of LPS to induction of effector and regulatory responses by HDEs. Shown are mean cell numbers ± SEM of the indicated leukocyte subsets in BAL. (A) Dose response for HDEs. Mice were sensitized with OVA together with indicated amounts of HDEs and challenged on a single occasion (open rectangles) or six occasions (solid rectangles). *a*, $P < 0.05$, OVA-HDE sensitized mice vs. 'OVA only' controls. *b,* $P < 0.05$, 20 µl vs. smaller volumes HDE. (B) Contribution of LPS component of HDEs to airway neutrophilia and eosinophilia. *Tlr4*-deficient ($Tlr4^{-/-}$) and WT mice were sensitized to OVA using 20 µl of the indicated HDE, and then challenged as indicated. Data are from one of 2 experiments giving similar results. $n = 6$ mice/group. *, $P < 0.05$. (C) Addition of LPS to 'endo lo' HDE inhibits eosinophilic inflammation. Mice were sensitized to OVA using the unmodified or LPS-supplemented HDE and challenged as indicated. Data are from a single experiment. $n = 6$ mice/group. *, $P < 0.05$.

Figure 6. Analysis of Tregs. (A) Analysis of total Foxp3$^+$ Tregs. Shown are the gating strategy used to identify CD4$^+$Foxp3$^+$ Tregs in *Foxp3^{gfp}* mice, as well as total numbers of Tregs in mice sensitized to OVA using the indicated dose of LPS and subsequently challenged on 6 consecutive days. (B) Gating strategy for OVA-specific OT-II (CD45.1) and nonspecific (CD45.2) Foxp3$^+$ Tregs. (C) Gating for Foxp3+ cells (top), as well as percent (middle) and number (bottom) of CD4$^+$ T cells that express Foxp3. (D) ICOS display on non-specific and OVA-specific Foxp3$^+$ Tregs. Gating for ICOS$^+$Foxp3$^+$ cells (top), numbers of CD4$^+$Foxp3$^+$ cells displaying ICOS (middle), and geometric means of fluorescent intensity for ICOS staining (GMFI ICOS) ± SEM (bottom). *, $P < 0.05$. Data are from one of 2 experiments giving similar results. n = 4-5 mice/group.

Figure 1

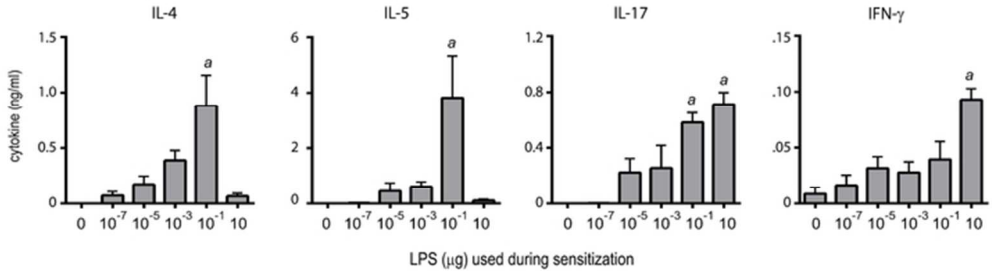

Figure 1
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Figure 2

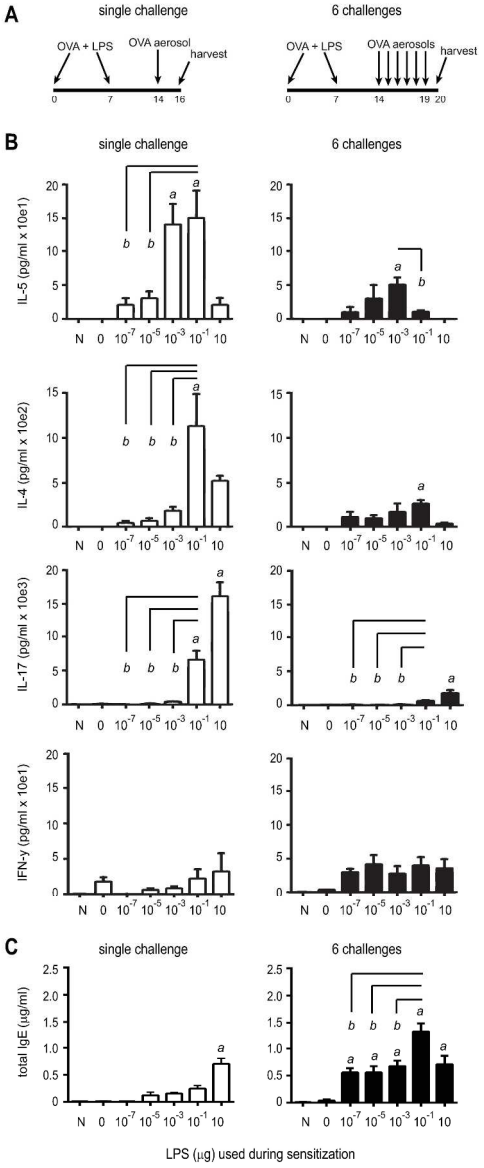

Figure 2

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Figure 3

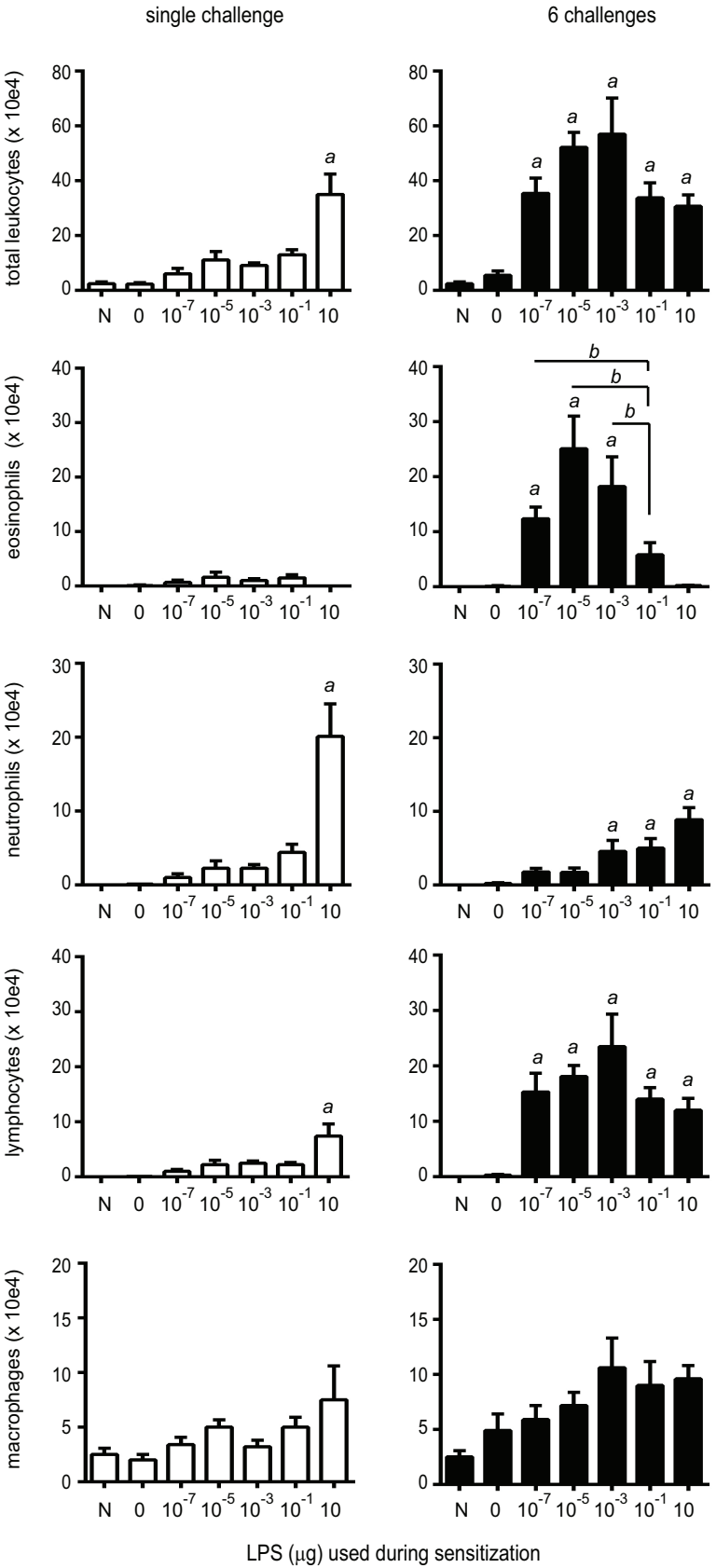
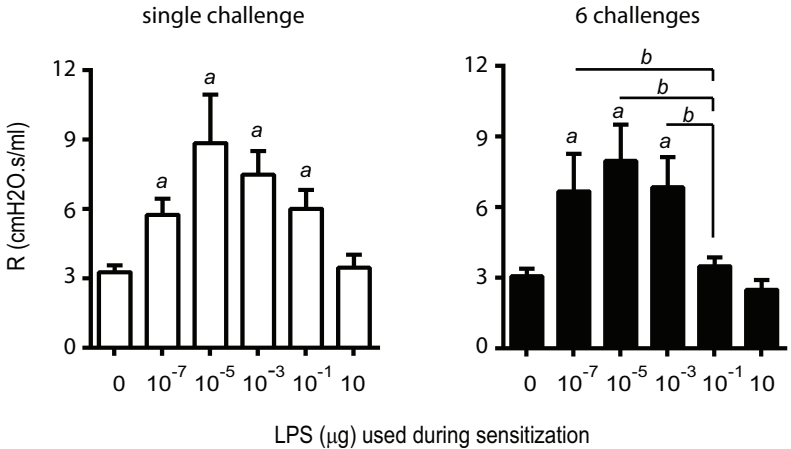

single challenge
6 challenges
total leukocytes (x 10e4)
eosinophils (x 10e4)
neutrophils (x 10e4)
lymphocytes (x 10e4)
macrophages (x 10e4)
N 0 10-7 10-5 10-3 10-1 10
LPS (μg) used during sensitization

Figure 4


single challenge
6 challenges
R (cmH2O.s/ml)
12
9
6
3
0
a
b
0
10-7
10-5
10-3
10-1
10
LPS (μg) used during sensitization

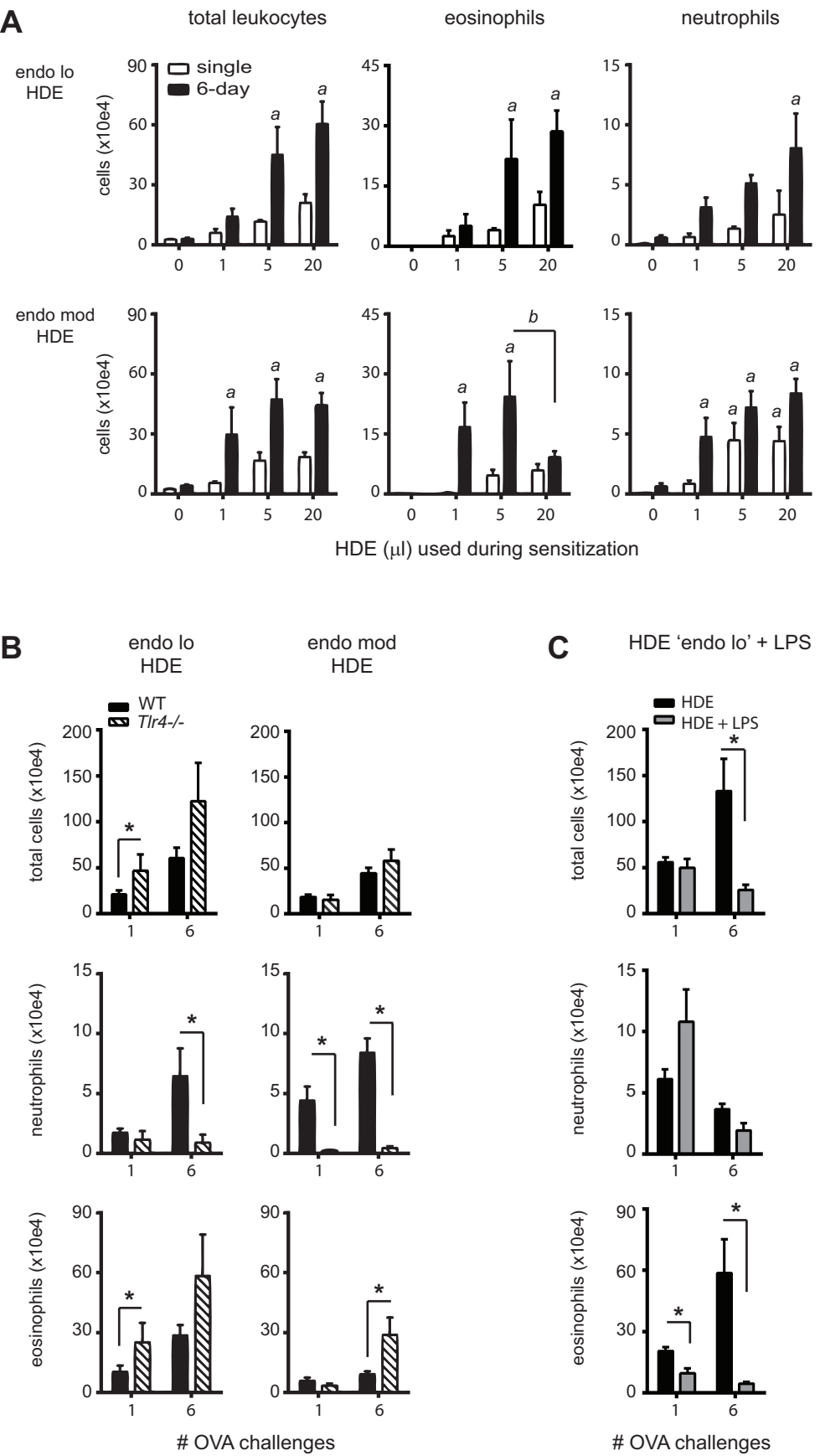
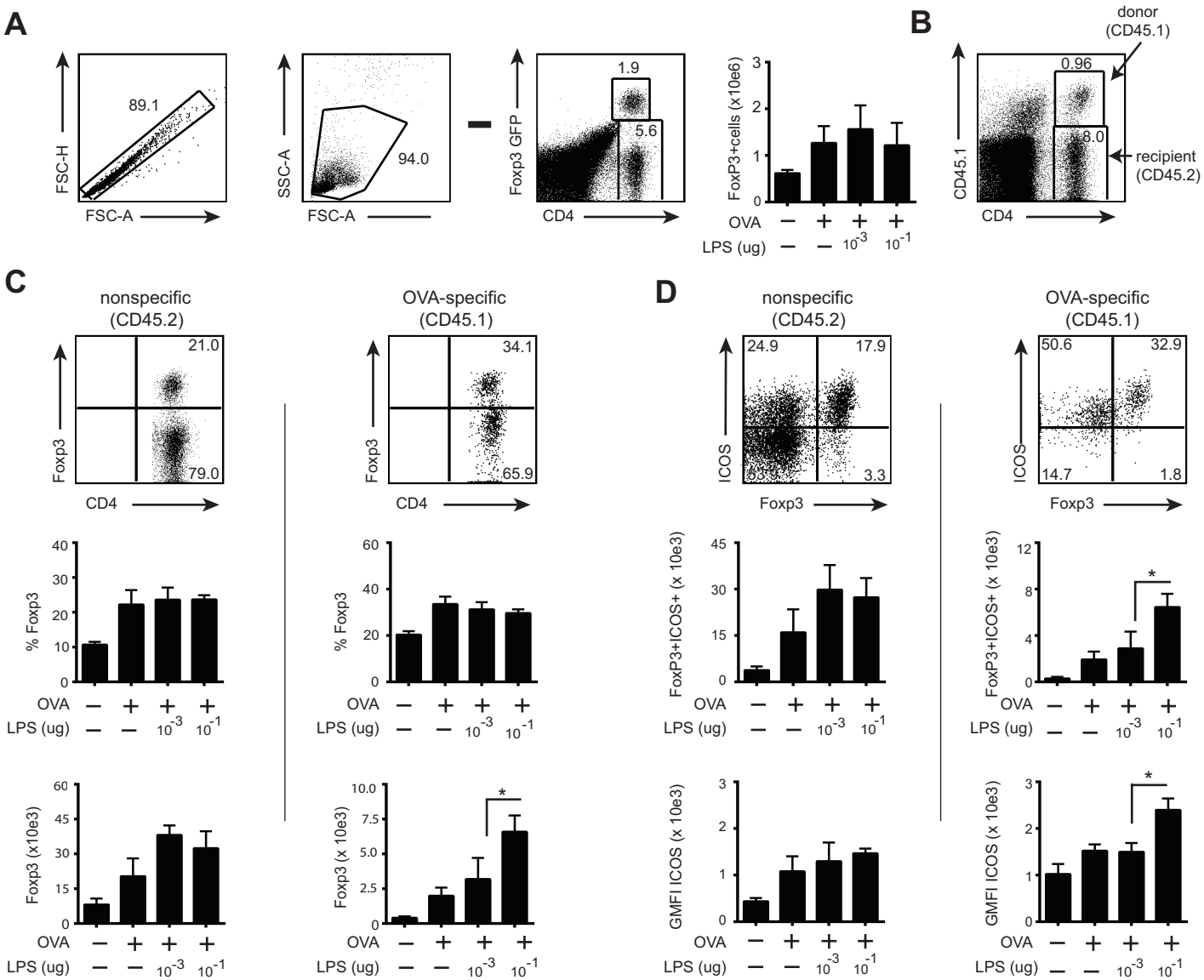

A
total leukocytes
eosinophils
neutrophils
endo lo HDE
endo mod HDE
single
6-day
cells (x10e4)
a
b
HDE (μl) used during sensitization
B
endo lo HDE
endo mod HDE
WT
Tlr4-/-
total cells (x10e4)
neutrophils (x10e4)
eosinophils (x10e4)
OVA challenges
C
HDE 'endo lo' + LPS
HDE
HDE + LPS
total cells (x10e4)
neutrophils (x10e4)
eosinophils (x10e4)
OVA challenges

Figure 6


A
89.1
FSC-H
FSC-A
94.0
SSC-A
FSC-A
1.9
5.6
Foxp3 GFP
CD4
FoxP3+cells (x10e6)
OVA
LPS (ug)
B
donor
(CD45.1)
0.96
8.0
recipient
(CD45.2)
CD45.1
CD4
C
nonspecific
(CD45.2)
21.0
79.0
Foxp3
CD4
OVA-specific
(CD45.1)
34.1
65.9
% Foxp3
Foxp3 (x10e3)
Foxp3 (x 10e3)
D
nonspecific
(CD45.2)
24.9
17.9
3.3
ICOS
Foxp3
OVA-specific
(CD45.1)
50.6
32.9
14.7
1.8
FoxP3+ICOS+ (x 10e3)
GMFI ICOS (x 10e3)